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Modulators of Response to Tumor Necrosis-Factor-related Apoptosis Inducing Ligand (TRAIL) Therapy in Ovarian Cancer

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14. ABSTRACT

Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. We have previously identified a homeobox gene, Six1, which is overexpressed in ovarian cancers as compared to normal ovarian surface epithelium. Overexpression of six1 is associated with resistance to Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) based therapies. We have discovered elevated Six1 is associated with tumor formation in mice and that the TRAIL decoy receptor DcR2 is overexpressed when Six1 is overexpressed. However, knockdown of DcR2 does not restore TRAIL sensitivity to Six1 overexpressing tumors, implying additional mechanisms. On-going studies are evaluating the mechanism and significance of thee findings on the way to designing new treatments for ovarian cancer.

15. SUBJECT TERMS
Ovarian cancer, TRAIL, TNF, Homeobox gene, Six1

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INTRODUCTION:

Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. Most ovarian cancers are diagnosed late and current treatment results only in a 20% 5-year survival in advanced disease. More effective therapies are urgently needed. One of the most promising therapies in development for ovarian cancer is the use of either the Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) or agonistic antibodies that activate the receptors for TRAIL. Both these strategies are designed to induce apoptosis in ovarian cancer cells. TRAIL therapies are particularly exciting because TRAIL reverses chemoresistance to standard chemotherapy as well as having a direct growth inhibitory effect on ovarian cancer cells, while sparing normal ovarian cells. However, the characteristics of ovarian tumor cells that determine whether TRAIL pathway agonists will be effective are poorly understood. For this reason, we currently do not have a rational basis for selecting patients who will benefit most from drugs that target this pathway or for improving the clinical response in those patients whose tumors are refractory to TRAIL pathway activators.

We have previously identified a homeobox gene, Six1, which is over-expressed in ovarian cancers as compared to normal ovarian surface epithelium. Expression of Six1 is correlated with poor clinical prognosis and confers resistance to TRAIL, possibly via upregulation of a decoy receptor. Our original hypothesis was that **"Six1 expression in ovarian cell lines and primary tumor cells results in resistance to TRAIL-induced apoptosis through activation of the DcR1 decoy receptor".** In the first year of the award, DcR1 expression in relation to various Six1 over-expression systems was evaluated and was not found to correlate with Six1 over-expression. However, a related TRAIL decoy receptor, DcR2 was found to increase in Six1 over-expressing cells, and in the second year of the award, further study of this mechanism was reported.

Hence, the specific aims are as follows: (1) to confirm *DcR2* as a downstream target of Six1 in ovarian cancer cells, (2) To determine if *DcR2* expression is the mechanism by which Six1 regulates the response of ovarian cancer cells to TRAIL pathway agonists, and (3) To determine if Six1 expression regulates the response of cell lines derived from primary ovarian cancers to TRAIL pathway agonists. These specific aims are identical to those in the original proposal with the exception of the substitution of DcR2 for DcR1. This document is the year three report. A no-cost extension has been approved and the final report will be submitted in April 2011.

In year two of the award (see 4/30/2009 report for details), we analyzed DcR2 as a downstream target of Six1. We showed that DcR2 mRNA was associated with increased Six1 mRNA expression in a panel of 15 ovarian cancer cell lines (mean DcR2 58±12 ag/ng 18srRNA for Six1 below the sample mean versus 127± 36 ag/ng 18s rRNA for Six1 at or above the sample mean, p=0.045 t-test). We had demonstrated an association between Six1 over expression and increased DcR2 expression in year one, but we were not able to demonstrate an association between Six1 knockdown from over-expressing cell lines and decreased DcR2 in year two. Although knockdown of DcR2 did restore a small (and statistically significant p=0.02) sensitivity to TRAIL in the Six1 over

expressing A2780 ovarian cancer cell line, the magnitude of the induction of sensitivity was much less that that expected (as comparison to induction of sensitivity from Six1 knockdown). A Six1 knockdown model for the SKOV3 cell line had been prepared in year two but not yet analyzed in regard to TRAIL sensitivity and these results are reported this year.

In year two, we reported that we had enrolled 29 patients, established 17 primary ovarian cancer cell lines from those patients, and tested the cell lines for tumorigenicity in CB-17 SCID mice, Six1 expression and TRAIL sensitivity. 6/17 cell lines generated tumors and tumorigenicity was correlated with increased Six1 expression (χ^2 p=0.02). Although there was no correlation between Six1 level and TRAIL sensitivity in the primary tumors, we discovered that primary cell lines as well as the majority of mice tumors lose Six1 expression with in-vitro and in-vivo passage, hence the Six1 level of the tumors is not reflective of the Six1 level of the cell line at the time it is being tested for TRAIL sensitivity. We discussed numerous strategies for completing this task in the year two report and these are further discussed in this report. Additionally, we have developed an *ex-vivo* system and will complete these studies in year four.

Because our experiments were designed to generate data that would be helpful in the design of phase I studies of TRAIL and its agonistic antibodies in cancers (ovarian and others), we are performing additional experiments aimed at the mechanism of TRAIL resistance in Six1 over-expressing cells. We are underway to predicting which cancers are TRAIL insensitive by virtue of their levels of Six1 expression, providing a way to select patients for TRAIL clinical trials that are more likely to benefit from this therapy. Furthermore, many currently used chemotherapeutic agents exert their cytotoxic effect through activating the TRAIL pathway and TRAIL therapy is synergistic with many chemotherapies. Hence, TRAIL resistance may be a marker for chemotherapy resistance and over-coming TRAIL resistance may render cells sensitive to chemotherapy. Since development of chemoresistance is a major obstacle to successful ovarian cancer therapy, a natural extension of our findings in a subsequent proposal would be to study the effects of reversing TRAIL resistance on the effectiveness of chemotherapy.

BODY:

The following section is organized according to the proposed statement of work for the first second and third years of the award and accomplishments towards completing the task.

Task 1. Verify DcR1 (DcR2) as a target of Six1 (1-9 months) – Completed.

As noted per the year one report, this task was modified to study DcR2 due to the lack of correlation between DcR1 and Six1 and initial data showing a positive correlation between DcR2 and Six1. Hence the specific tasks became:

a. Collect and propagate specimens and cell lines to complete Six1 RNA and *DcR2* RNA and protein analysis.

b. Perform CaOV3-Six1 and SKOV3 SiRNA experiments.

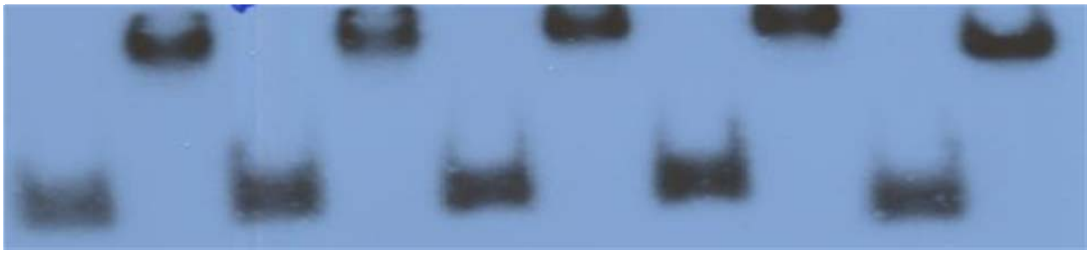
This task was completed and reported on the year 2 report**.**

Task 2. **Determine whether *DcR2* is a direct or indirect target of Six1.**

a. Gel shift (electrophoretic mobility assay)
b. Chromatin IP experiments
c. Promoter activation studies

This task was reported as completed in the year two report. Additional experiments were in progress at the time of the year 2 report and are added for completion.

To analyze the likelihood of DcR2 promoter binding by Six1, a 2000 bp sequence of the DcR2 mRNA upstream of the DcR2 translation start site was examined for the presence of the "TCAGG/CCTGA" consensus Six1 binding sequence[1] and 4 such sequences were found. Oligonucletodies (30 bp) of these regions were prepared and are listed in table 1. Results of the electrophoretic mobility assay are shown in Figure 1. An MEF3 site known to bind and gel-shift extracts of purified Six1 protein is shown as positive control. All the TCAGG sites in the DcR2 promoter region bound Six1 protein and shifted its movement on the gel, suggesting an interaction and supporting previously reported data.



-------#1------- --------#2-------- -------#3------- -------#4------- -----MEF3-------

Figure 1. Gel-shift shows binding of 4 oligonucleotides containing consensus sites in DcR2 promoter to Six1 purifed protein. Sequences for oligonucleotides 1-4 and MEF3 (consensus sequences in shaded box) are found in table. For each oligonucleotide, the first lane is the unbound negative control (probe) and the second lane is the bound oligonucleotide/protein complex showing the change in mobility. MEF3 is known to bind Six1 and is used as positive control.

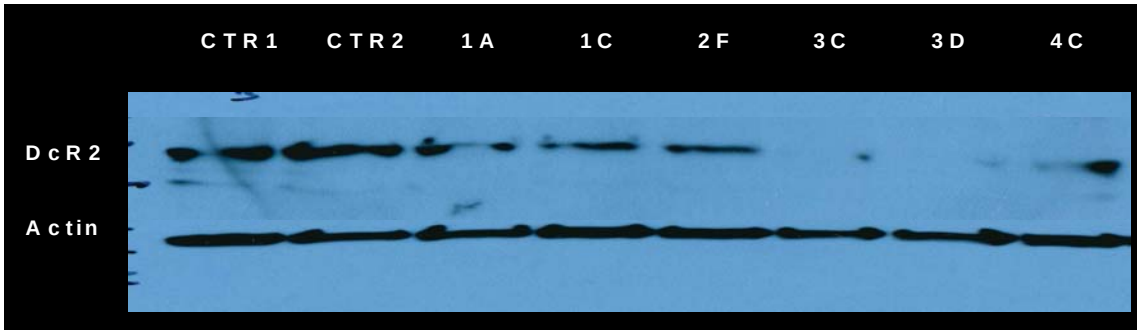
Table 1. Oligonucleotide sequences for Figure 1.

#1 TCA GTC TTC CTG AAG TCC CTG ACC TTT CAC (starting at bp 108)
#2 ACC ATG TGA GGG GTC AGG AGC CGA CTC ATC (starting at bp 1018)
#3 GCC AGG AAG TAG TTC AGG GTT TAA GAA GAG (starting at bp 1624)
#4 GGA GGG AGC AGG CTC AGG ATG GGC CTC CAG (starting at bp 1672)
MEF3 GGG GGC TCA GGT TTC TGT GGC

This data confirms our revised original hypothesis "The TRAIL decoy receptor DcR2 is a downstream target of Six1 in ovarian cancer cells".

We also initiated DcR2 knockdown experiments to analyze if the same phenotype seen with Six1 knockdown could be recreated with DcR2 knockdown. This would give functional relevance to any Six1/DcR2 interaction we would find. DcR2 knockdown in the cell line A2780 was reported in year two and resulted in a statistically significant, but modest sensitization to ETR2. There was no effect on TRAIL, Fas or ETR1 sensitivity. In our previously published manuscript, Six1 knockdown sensitized SKOV3 cells to TRAIL. Accordingly we proceeded to analyze if DcR2 knockdown would sensitize SKOV3 cells to TRAIL. The SureSilencing (SABiosciences, Frederick, MD) shRNA knockdown system was used to generate multiple SKOV3 DcR2 knockdown cell lines using 4 different primer sets (numbered 1-4) and controls. A western blot of DcR2 with controls CTR1 and CTR2, 2 clones from primer sets 1 (1A,1C) and 3 (3C,3D) and a clone from primer sets 2 (2F) and 4 (4C) are shown in Figure 2. DcR2 was decreased in the knockdown clones compared to controls.

Figure 2. DcR2 western blot of SKOV3 control clones (CTR1 and CTR2) and shRNA knockdown clones (1A,1C,2F,3C,3D and 4C) with β-Actin loading controls.



The effect of DcR2 knockdown on sensitivity to TRAIL and agonistic TRAIL antibodies was then studied by performing dose-response assays to TRAIL, FasL and ETR1 and ETR2 using control and DcR2 knockdown clones. Results are shown in Figure 3.

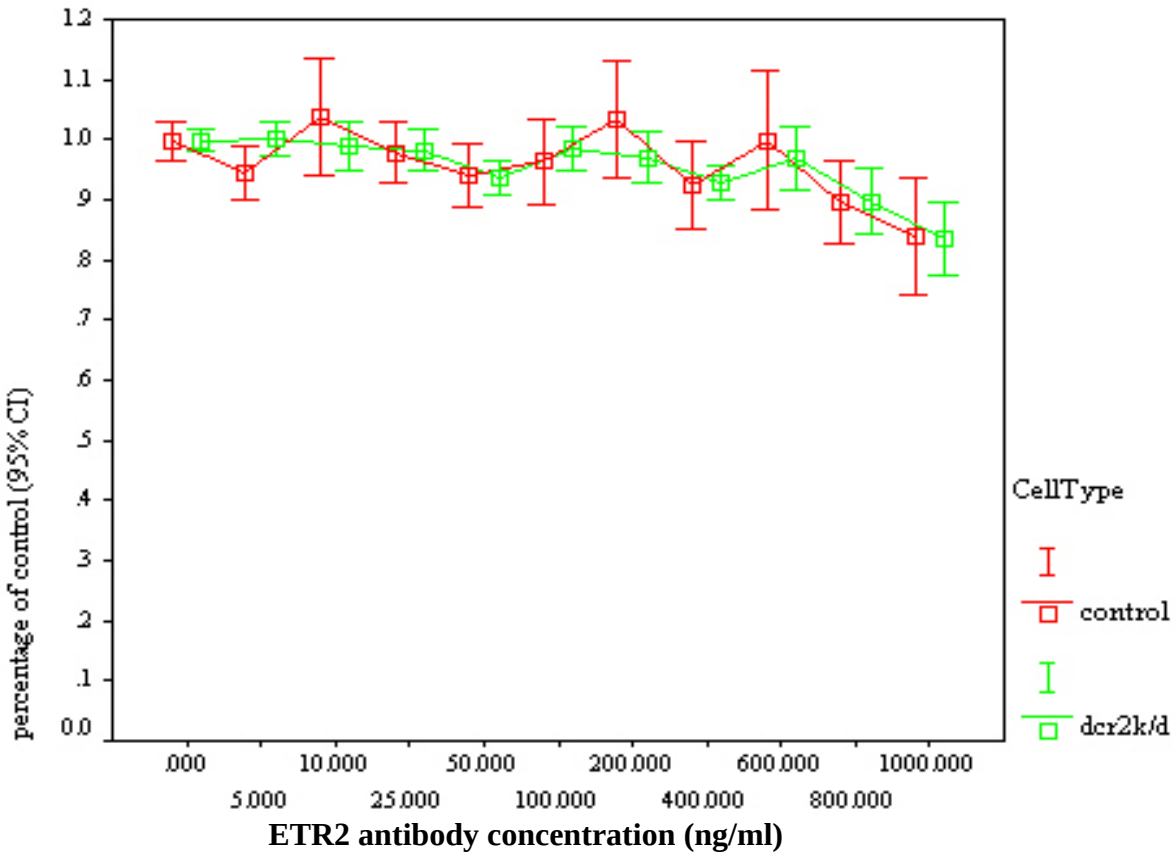


Figure 3. DcR2 knockdown does not sensitize SKOV3 cells to ETR2. Dose-response to the agnostic antibody to TRAIL-DR5, ETR2 are plotted as percent of control growth (± 95% Confidence Interval-CI) for the SKOV3 control cell line and the shRNA DcR2 knockdown clones 1A, 1C, 2F, 3C, 2D, 3D and 4C pooled together.

Overall, these studies show that DcR2 is a downstream target of Six1, but that manipulating DcR2 is not likely to have any effect on the sensitivity of cells either to TRAIL or its receptor agonists. To further analyze the relationship between Six1 expression and TRAIL receptor expression, we studied TRAIL DR4, DR5, DcR1 and DcR2 cell surface receptor expression by flow cytometry in a panel of 15 ovarian cancer cell lines with Six1 levels ranging between 0 fg Six1/ng 18s rRNA to 763 fg Six1/ng 18s rRNA. Data is shown in Figure 4. As expected, increased Six1 correlated with increased DcR2 (Kruskal-Wallis test p=0.05), but not DcR1 or DR4. Interestingly, Six1 expression was also associated with increased DR5 (Kruskal-Wallis test p =0.002). Even though DcR2 was increased with increased Six1, the concomitant DR5 increase was to the extent that the DR5/DcR2 ratio was also *greater* with increased DcR2 (we would hypothesize that it would be less, ie., more DcR2 as compared to DR5) implying that that the increase in DcR2 by Six1 may be compensated for by increased DR5 and that increased DcR2 may not be the mechanism for TRAIL resistance via receptor competition. These experimental observations would need to be further verified. Given this data, additional promoter activation studies and chromatin IP studies were not pursued. While this task is completed, additional analysis of TRAIL pathway components downstream of the

receptors and the DISC complex in ovarian cancer, which may shed light on the Six1-mediated changes in the TRAIL pathway, and where manipulation of the pathway components can be used as therapy, is on-going in our laboratory.

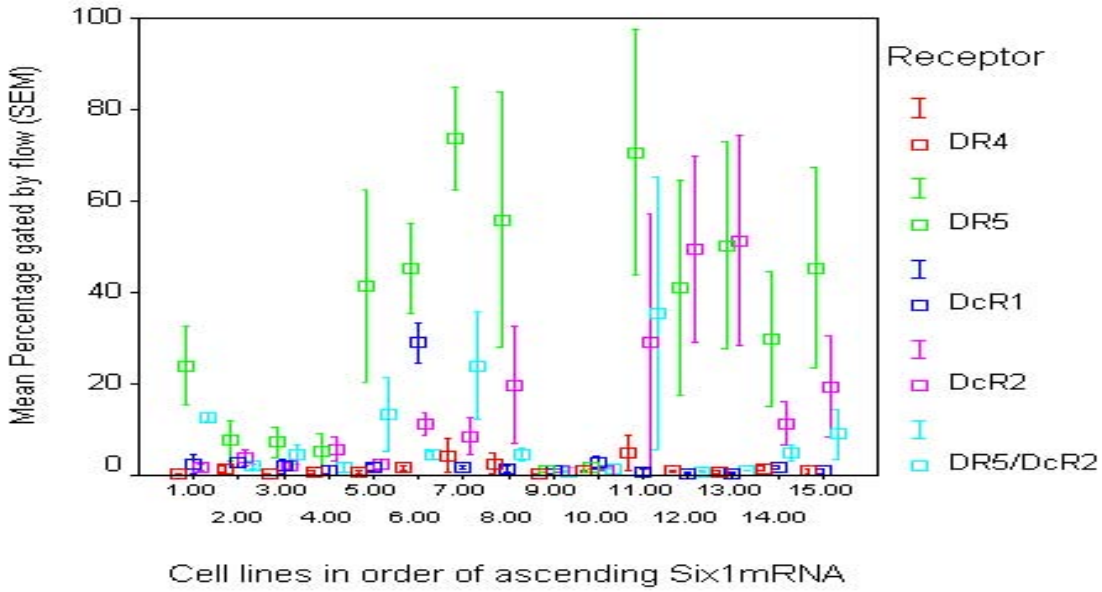


Figure 4. Analysis of TRAIL receptor level by flow cytometry in a panel of ovarian cancer cells with increasing levels of Six1. DcR2 mean percent positive cells by flow cytometry (± standard error of the mean SEM of 3-8 repeats) are low in the first five cell lines, but are increased in 8/10 subsequent cell lines. However, DR5 levels are similarly increased. The cell lines are 1. OV433 (0 fg Six1/ng 18srRNA), 2. OV432 (0 fg Six1/ng 18srRNA), 3. OV420 (0 fg Six1/ng 18srRNA), 4. OVCAR5 (5 fg Six1/ng 18srRNA), 5. OV2008 (17 fg Six1/ng 18srRNA), 6. DOV13 (19 fg Six1/ng 18srRNA), 7. SNU251 (36 fg Six1/ng 18srRNA), 8. OVCAR2 (81 fg Six1/ng 18srRNA), 9. OV1847 (85 fg Six1/ng 18srRNA), 10. CaOV3 (89 fg Six1/ng 18srRNA), 11. PECOC167 (109 fg Six1/ng 18srRNA), 12. HeyC2 (121 fg Six1/ng 18srRNA), 13. Hey (143 fg Six1/ng 18srRNA), 14. SKOV3 (155 fg Six1/ng 18srRNA), 15. A2780 (763 fg Six1/ng 18srRNA).

Task 3. **Evaluate TRAIL panel sensitivity in Six1 over-expressing and knock-down cells**

A. Generate inducible models of Six1 expression.
B. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using existing Six1-CaOV3 over-expression model and Six1 knockdown model, save cell pellets and extract RNA and protein.

In the past year, multiple systems were used to generate both inducible over-expression and inducible knockdown. These included the BD RevTet tetracyclin-on overexpression system and the P30ETREMIRAG lentiviral knockdown system. Neither yielded reproducible and tightly controlled effects as required for this task. While expected to

have baseline levels of Six1, controls from the BD RevTet system also over-expressed Six1 suggesting that the system was either "leaky", or the control media contained amounts of tetracycline sufficient to induce the transgene without the addition of any tetracycline. Certified tetracycline free media was ordered and the clones were re-isolated and grown. However, the transgene was expressed, even tetrascycle-free media. The lentiviral knockdown system generated verified expression of the transgene as noted by the presence of a GFP tag on the selected clones, however, Six1 could not be suppressed.

To complete this task in the absence of an inducible model, additional stable CaOV3-Six1 over-expressing clones were generated using pcDNA3.1 plasmid transfection. A western blot for Six1 expression for the control (CAT-a1 and CAT-b1) and the Six1 overexpressing clones Six1-c1, Six1-c2, Six1-d1, Six1-d2 and Six1-e is shown in Figure 5 and shows successful stable Six1 expression in the transfected clones.

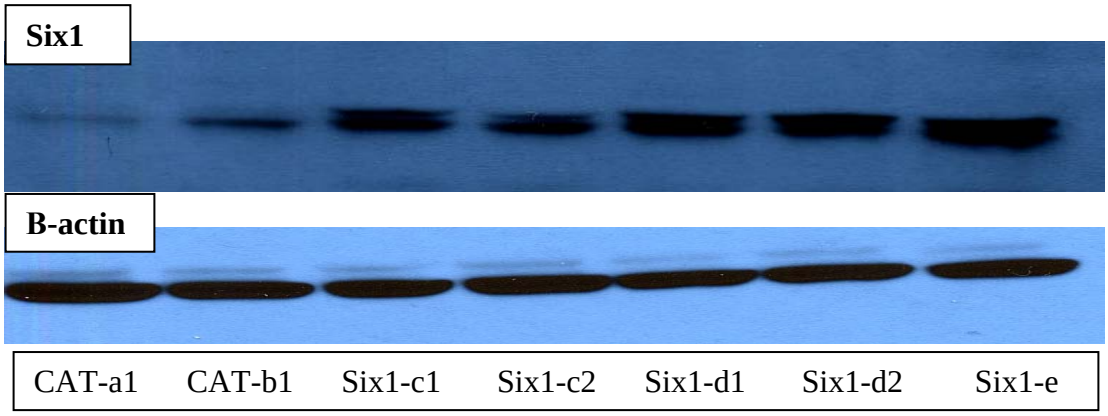


Figure 5. Generation of new CaOV3 Six1 over-expressing clones. A Western blot shows Six1 over-expression in the Six-c1, Six-c2, Six-d1, Six-d2 and Six-e clones as compared to the CaOV3 CAT-a1 and CAT-b1 clones.

TRAIL sensitivity was assayed in the clones above using by performing dose-response curves using the MTS assay. Six1 overexpression was confirmed to result in TRAIL resistance as shown in Figure 6. IC_{50} values for Six1 over-expressing clones were 4-10 fold greater than in the CaOV3-CAT clones. This confirms our original hypothesis "Six1 overexpression in the CaOV3 ovarian cancer cell line blunts the response to TRAIL".

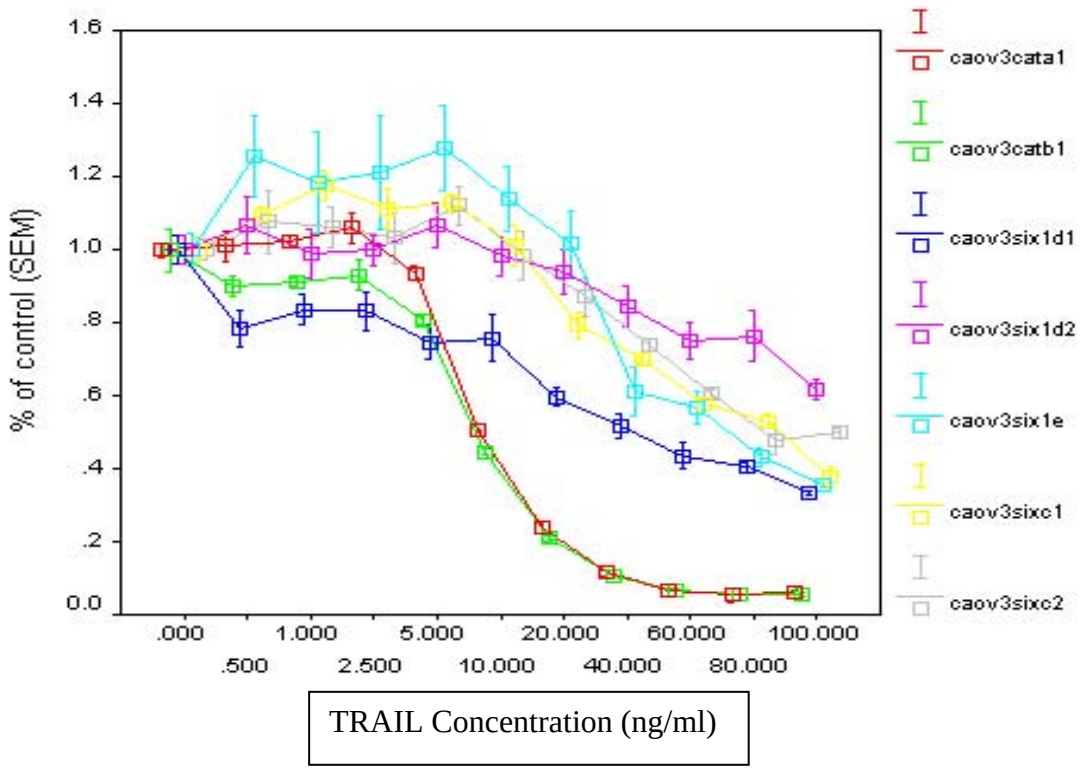


Figure 6. CaOV3 Six1 over-expressing clones develop resistance to TRAIL IC50 values are 10 ng/ml TRAIL for the CATa1 and CATb1 clones, 40 ng/ml TRAIL for the Six1-d1 clone, 80 ng/ml TRAIL for the Six1-c1, Six1-c2 and Six1-e clones and greater than 100 ng/ml TRAIl for the Six1-d2 clone.

ETR1 sensitivity was assayed in the clones above using by performing dose-response curves using the MTS assay. Results are presented in Figure 7. ETR1 sensitivity was seen in the CaOV3-CAT-a1 clone but not in the CaOV3-Catb1 clone or any of the CaOV3-Six1 clones. ETR2 sensitivity was assayed and showed a similar pattern (data not shown). The difference between TRAIL sensitivity and ETR1/ETR2 sensitivity in the CaOV3-CATb1 clone may be related to the slightly greater expression of Six1 in the CaOV3-b1 clone as compared to the CaOV3-CATa1 clone and implies a different threshold for TRAIL sensitivity as compared to TRAIL receptor agonist sensitivity. This experimental observation would need to be verified by repeated testing of these clones. All clones were resistant to FasL with IC_{50} > 5000 pg/ml.

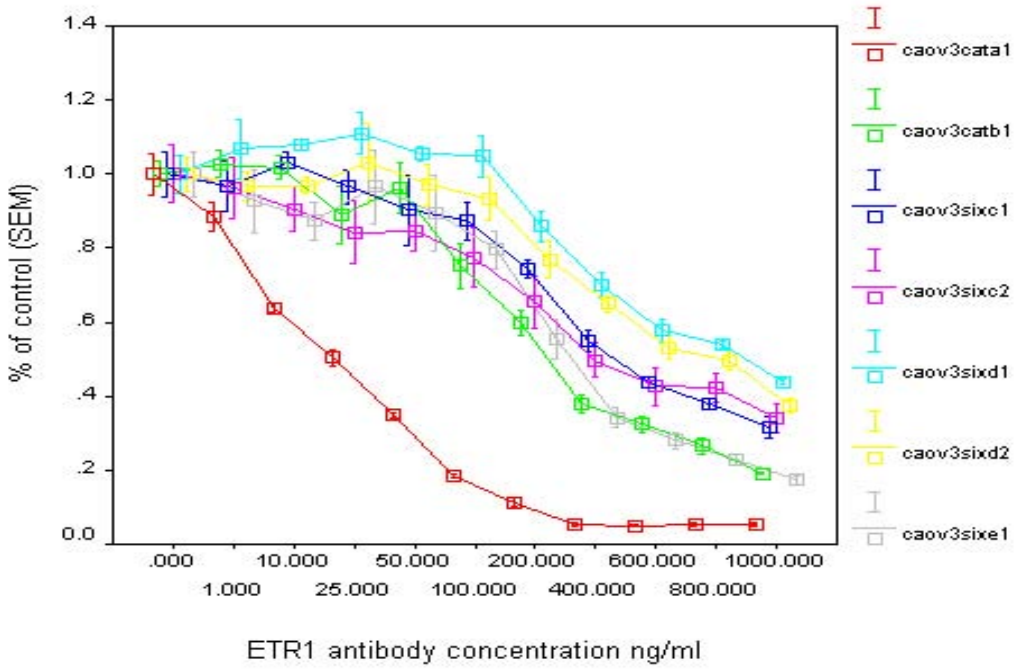


Figure 7. CaOV3 Six1 over-expressing clones develop resistance to ETR1. IC_{50} values are 10 ng/ml ETR1 for the CATa1 clone, 200 ng/ml ETR1 for the CAT-b1 clone, 250-300 ng/ml ETR1 for the Six1-c1, Six1-c2 and Six1-e1 clone, and 900 ng/ml ETR1 for the Six1-d1 and Six1-d2 clones.

Preference was given to DcR2 knockdown experiments rather than repeat of the Six1 knockdown experiments (as previously reported[2]) after completion of tasks 1 and 2 revealed DcR2 to be a relevant target. Results of DcR2 knockdown experiments are reported under task 2.

Task 4. **Evaluate TRAIL panel sensitivity in primary ovarian cancer cell lines and correlate with Six1 and *DcR2* expression.**

a. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using primary ovarian cancer cell lines, save cell pellets.
b. Extract RNA and protein from cell pellets, correlate with Six1 and DcR2 expression

In year two, we reported that we had established cell lines from patients with ovarian cancer and tested sensitivity to TRAIL and TRAIL agonistic antibodies. Twenty-nine patients had been enrolled and 17 specimens had generated cell lines that could be assayed. All patient specimens were resistant to FasL up to 5000 pg/ml. 6/17 cell lines generated tumors in CB-17 SCID mice and tumorigenicity was associated with tumor Six1 expression ($p=0.02$ χ^2). We reported no clear developing correlation between Six1 status and TRAIL resistance in primary cell lines derived from patient tumors, but subsequently discovered that some primary cell lines rapidly lose Six1 expression in culture and that the cells being tested for TRAIL sensitivity may not be similar to those growing in the patient. Hence we repeated the analysis using the established cell line

Six1 mRNA level rather than the original tumor Six1 mRNA level. Results are presented in Table 2. No correlation was found between primary ovarian cancer cell line Six1 mRNA expression (none versus any) and TRAIL or ETR2 resistance in primary cell lines generated from patients with metastatic ovarian cancer ($p= 0.25$ $\chi 2$ test).

Table 2. Primary cell lines isolated from patients with Age of the patient, Histology of the primary tumor, cell line Six1 level, tumorigenicity in CB-17 SCID mice and TRAIL, ETR1 and ETR2 IC_{50}. Resistance was defined as greater than 50 ng/ml for TRAIL and greater than 1000 pg/ml for ETR1 and ETR2. All cell lines were resistant to FasL up to 5000 pg/ml.

#	Age	Stage	Histology	Cell line Six1 fg/ng 18s rRNA	Tumors?	TRAIL IC_{50}	ETR1 IC_{50}	ETR2 IC_{50}
141	75	IIIc	Serous	0	No	R	R	R
140	48	IIIc	Serous	0	No	R	R	R
137	58	IIIc	Serous	0	No	R	R	R
142	84	IV	Serous	0	No	R	R	R
139	56	IIIc	Serous	0	No	R	R	R
138	52	IIIc	Endo	0	Yes	R	R	R
163	52	IIIc	Serous	0	No	2.5 ng/ml	R	200 pg/ml
158	45	IIIc	Serous	1	Yes	5 ng/ml	R	600 pg/ml
162	57	IIIc	Serous	7	No	R	R	R
173	59	IIIb	Serous	7	Yes	R	R	R
150	65	IIIc	Serous	9	Yes	1 ng/ml	R	200 pg/ml
153	71	IV	Serous	24	No	R	R	R
161	52	IV	Serous	66	No	5 ng/ml	R	400 pg/ml
160	60	IV	Mucinous	179	No	R	R	R
167	47	IV	Mixed	220	Yes	R	R	R
159	43	IIIa	Clear Cell	311	No	R	R	R
164	52	IIIc	Serous	324	Yes	1 ng/ml	R	350 pg/ml

While these results do not support our hypothesis, we recognize that cell lines obtained from patient tumors may not be representative of the *in-vivo* tumor. Hence we have subsequently pursued a novel *ex-vivo* method to directly assay TRAIL sensitivity in relation to Six1 status in patient tumors. Preliminary experiments show our ability to harvest tissues and process them using the Krumdieck tissue dissector (Alabama Research and Development), to maintain tissues *ex-vivo*, to study proliferation using standard MTS assay (CellTiter 96® AQ_{ueous} Assay, Promega) in the *ex-vivo* system, and to assay proliferation, and detect apoptosis. To demonstrate ability to study proliferation, we treated 300 μm slices of tumor from a 58 year old patient with type II (high grade serous) platinum sensitive recurrent ovarian tumor and omental metastases. A

2cm by 2 cm section of omental tumor was cored under sterile technique and sliced using the Krumdieck tissue slicer with sterile PBS in the flow chamber. Slices were transferred to 24 well plates and covered with 4 ml of RPMI1640 media. After 24 hours, media was changed to control (media + vehicle) or cisplatin at 50 µM or cisplatin + TNF-related apoptosis inducing ligand (TRAIL) at 50 ng/ml or TRAIL alone. After 3 hours, the MTS solution was added to the media for 4 hours. A 500 µl aliquot was pipetted into each of 6 wells of a 96 well plate and read using an ELISA plate reader at 490 nm. Results (Figure 8) are reported as percent of media only controls. Six1 mRNA was assayed by qRT-PCR and revealed a low level of 2 ag Six1/fg 18s rRNA (SKOV3 reference 114 ag Six1/fg 18s rRNA). This low Six1 expressing tumor was sensitive to TRAIL as well as to Cisplatin.

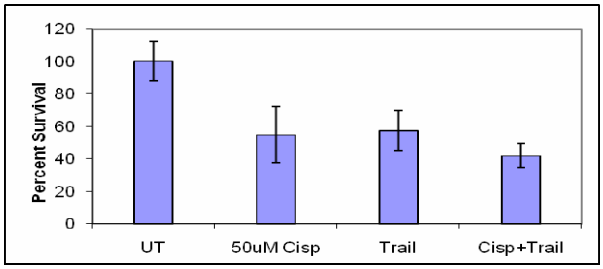


Figure 8. MTS assay from ex-vivo culture can detect proliferation differences. Tissue slices were treated with cisplatin, TRAIL, and cisplatin + TRAIL (reported as percent of control untreated-UT), 50 µM cisplatin or 50 ng/ml TRAIL resulted in 60% of control proliferation. Addition of TRAIL to cisplatin did not decrease this further.

Following the MTS assay, the slices were washed, fixed in 10% formalin and paraffin embedded. 8 µm slices were cut and placed on Colormark Plus slides (Erie Scientific). Slides were assayed for proliferation by Ki67 using 1:1000 dilution of SP6 antibody (Thermo scientific) and developed using the Ventanna DAB kit using a Ventana NexES IHC autostainer. Staining was quantitated by counting brown (stained) cells versus blue (Hematoxylin counterstain) cells. Apoptosis was assayed using the Apotag® Red In Situ kit (Chemicon International). Cells undergoing apoptosis (pink or lighter color) and counterstained (DAPI-blue or darker color) were counted. Results are reported in Figure 9.

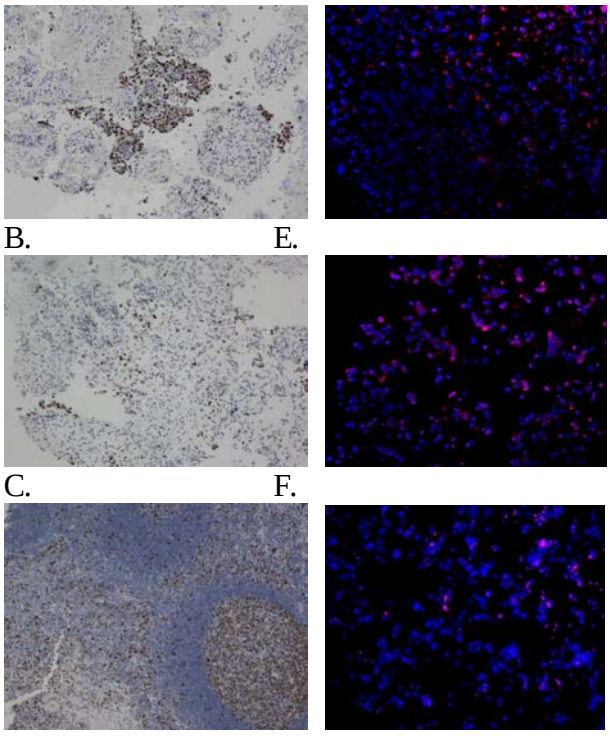


Figure 9. Proliferation and apoptosis staining of ex-vivo slices of serous ovarian cancer treated with cisplatin or cisplatin + TNF-related Apoptosis Inducing Ligand (TRAIL). A-C. Ki67 staining (dark) is present with cisplatin treatment (A) and decreased upon the addition of TRAIL (B). Normal tonsil is used as positive control (C). D-F. Cells undergoing apoptosis (pink or lighter color) with cisplatin treatment (D) and increased with the addition of TRAIL (E). The same slice pre-treated with DNAse1 is used as control(F).

In the coming year, we will use this *ex-vivo* system to complete this ask and verify or refuse the hypothesis "Overexpression of Six1 in ovarian cancer correlates with the responsiveness of the cancer to TRAIL mediated apoptosis".

Task 5. **Establish Syngeneic (mouse) Six1 over-expression Model**

In year two, it was discovered that only one of ten mouse ovarian cancer cell lines expressed Six1. Six1 expression is most likely not an important contributor to mouse ovarian cancers and given our development of *ex-vivo* models for studying primary human ovarian tumors, development of a syngeneic mouse model was not pursued.

Task 6. **Xenograft and/or syngeneic model Six1/*DcR2* over-expression and knockdown analysis.**

a. Test TRAIL, Etoposide (instead of FasL), ETR1 and ETR2 response in xenograft or syngeneic model
b. Evaluate phenotype of in-vivo Six1/*DcR2* knockdown

Our first experiment was to study growth rates of CaOV3 CAT and CaOV3-Six1 transfectants on the flanks of 4-6 week old CB-17 SCID mice. 4 clones total, 4 mice/group and two tumors/ mouse were initiated by injecting 1 x 10^7 cells and observing for tumor growth with biweekly measurements of tumor size. Six1 expressing tumors measured 38±6 mm^3 at 2 weeks as compared to 7±3 mm^3 for CAT clones ($p<0.001$ t-test) demonstrating a faster initial growth rate for Six1 expressing tumors. However tumor growth was poor in the subsequent weeks for both CAT clones and Six1 clones. At 10 weeks Six1 expressing tumors measured 50±19 mm^3 as opposed to 29±11 mm^3 for CAT expressing clones. This difference was no longer significant. A subsequent experiment demonstrated that the CaOV3-Six transfectant xenograft tumors lost Six1 expression within 2 weeks, associated with a decrease in growth rate to baseline. Attempts to study the SKOV3 Six1 knockdown clones in the same system resulted in robust tumor growth in the SKOV3 parental line, but no tumor growth in the SKOV3 Six1 siRNA tumors. These findings were encouraging because they highlight the importance of Six1 in maintaining tumor growth. However, loss of Six1 over-expression and the lack of tumor growth in knockdown clones makes the study of the effects of treatment in this system difficult. Hence we have favored development of the *ex-vivo* model. Preliminary data in presented under task 4 and the bulk of these experiments will be completed during the coming year and reported in the final report.

KEY RESEREARCH ACCOMPLISMENTS:

- Six1 overexpression is associated with TRAIL resistance and over-expression of the TRAIL DcR2 decoy receptor in ovarian cancer cell lines and in a syngeneic over-expression system, however, manipulation of DcR2 does not seem to affect TRAIL

sensitivity, raising the possibility that Six1 may affect other TRAIL pathway components.
- Ovarian cancer cells express TRAIL DR4, DR5 and the decoy receptor DcR2. DcR1 expression is uncommon.
- Tumorigenicity in ovarian cancer cell lines and in Six1 over-expression and knockdown models appears to be related to Six1 expression.
- A new model has been developed to study Six1 related changes in proliferation and apoptosis in primary ovarian tumors

REPORTABLE OUTCOMES:

The following abstract was presented at a national meeting as a result of this research (pdf attached):

Qamar L, Syed N, Ford H, Thorburn A, Behbakht K. The Six1 homeobox gene is associated with TRAIL resistance in ovarian cancer and is correlated with increased Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) decoy receptor DcR2 in a Six1 overexpression model. Presented at the 40th Annual Meeting of the Society of Gynecologic Oncologists, published *Gynecol Oncol* 112(2009) page S158.

A manuscript entitled "TRAIL Receptor Signaling regulates chemosensitivity in vivo" was submitted for consideration of publication to Molecular Cancer Therapeutics on 4/26/2010. This manuscript addresses differences between in-vitro and in-vivo sensitivity to TRAIL. A pdf version of the final accepted manuscript will be submitted upon acceptance.

CONCLUSIONS:

Overexpression of the developmental homeobox gene Six1 is gaining importance as a mechanism for carcinogenesis and metastasis in an ever-growing list of malignancies. The list of downstream genes controlled by Six1 is also ever-growing and likely to be tissue specific. We have discovered that overexpression of the Six1 homeobox gene in ovarian cancer is associated with TRAIL resistance, but even though the TRAIL decoy receptor DcR2 is a downstream target of Six1, manipulation of DcR2 is unlikely to have therapeutic impact. We plan to conclude further mechanistic and functional studies in the last year of this proposal. These studies will point the way to strategies for reversing the effects of Six1 expression and potentially reversing chemoresistance by blocking the downstream targets of Six1 in ovarian cancers.

References:

1. Chai, L., et al., *Transcriptional activation of the SALL1 by the human SIX1 homeodomain during kidney development.* J Biol Chem, 2006. **281**(28): p. 18918-26.
2. Behbakht, K., et al., *Six1 overexpression in ovarian carcinoma causes resistance to TRAIL-mediated apoptosis and is associated with poor survival.* Cancer Res, 2007. **67**(7): p. 3036-42.

Conclusions: Clinicians struggle in their efforts to distinguish patients with recurrent ovarian cancer who have potentially reversible and treatable problems from those who are entering a terminal phase of their illness. In the final 100 days of an ovarian cancer patient's life, the disease produces distinct symptoms requiring management and resource utilization. Our data suggest that even as disease progresses, we are inclined to perform evaluations and offer treatments, as well as offer care to provide symptom management. Worsening gastrointestinal symptoms or increased use of hospital admission or procedures should identify patients as potentially moving toward the final phases of their illness.

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The search for meaning, symptoms and transvaginal ultrasonography screening for ovarian cancer: Predicting malignancy

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Objectives: The mortality rate of ovarian cancer is greater than that of all other major gynecologic malignancies. Most women present with advanced-stage disease, where response to treatment is limited and prognosis is poor. Detecting ovarian cancer at an early stage, when it is curable, has long been an important goal of gynecologic oncologists. Recently, it has been reported that certain symptom patterns can be informative for the presence of ovarian malignancy. The present investigation was performed to determine how well symptoms and ultrasound findings would predict ovarian malignancy individually or in combination.

Methods: A group of 450 women, all of whom received surgery due to participation in annual transvaginal ultrasonography (TVS) screening, were selected from 31,748 women enrolled. Symptom questionnaires were provided, and the tabulated results were compared with ultrasound reports and surgical pathology for 272 of the women.

Results: Thirty malignancies and 420 persisting benign tumors constituted the group under study. The ability to distinguish malignant from benign ovarian tumors was based on sensitivities, specificities, and ROC curve analysis. TVS performed better than symptom analysis for detecting malignancies (73.3% vs 20% sensitivity), and symptom analysis performed better for distinguishing benign tumors (91.3% vs 74.4% specificity). Decisions based on simultaneously meeting TVS and symptom criteria resulted in poorer identification of malignancy in ROC analysis (with Morphology Index (MI) >5 and symptom analysis, sensitivity=16.7%), but improved the ability to distinguish benign tumors (with MI>5 and symptom analysis, specificity=97.9%). Decisions based on satisfying either symptom criteria or TVS criteria had small increases in sensitivity (+3.3%) and coordinated small decreases in specificity (-5.8%).

Conclusions: Symptom analysis does identify malignant ovarian tumors, but its discrimination by itself is inferior to that of TVS. The clinical significance of the findings reported here is that: (1) a screen that is negative by both ultrasound and the symptom index is likely to indicate a benign tumor (specificity >97%), and (2) adding symptom information with equal weight as ultrasound slightly improves the discrimination of malignancy (one additional TP with a sensitivity increase=+3.3%). These results strongly indicate that the major screening benefit in discriminating malignancy is achieved via ultrasound tools, whereas symptom information can aid in reducing surgery on women with benign conditions that generate ultrasound abnormality. Combining symptom analysis with TVS improved the discrimination of benign tumors, but it is coordinated with much poorer discrimination of malignant tumors, indicating that informative symptoms can be expected to be absent in a large fraction (80%) of ovarian malignancies.

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The six1 homeobox gene is associated with resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in ovarian cancers and is correlated with increased TRAIL decoy receptor DcR2 in a six1 overexpression model

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Objectives: Ovarian cancers express TRAIL receptors and TRAIL synergizes with chemotherapy in ovarian cancers. However, up to 60% of ovarian cancers overexpress the Six1 homeobox gene and we have shown that Six1-overexpressing ovarian cancers are resistant to TRAIL. To assess the role of TRAIL decoy receptors in Six1-related TRAIL resistance, we studied the expression of TRAIL and TRAIL decoy receptors and correlated these with Six1 expression and dose response to TRAIL and TRAIL receptor agonists.

Methods: Six1 expression and TRAIL receptor DR4 and DR5 and decoy receptor DcR1 and DcR2 mRNA levels were analyzed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in a panel of 15 ovarian cancer cell lines as well as Six1 stable transfected CaOV3 clones and control CAT clones. Dose-response curves were generated to TRAIL, FasL (as a control for non TRAIL receptor-induced apoptosis)and agonistic antibodies to TRAIL DR4 and DR5 and correlated with Six1 and TRAIL receptor expression.

Results: All 15 cell lines expressed DR4 (mean=123±77 ag/ng rRNA), DR5 (mean=210±106 ag/ng rRNA) and DcR2 (mean=81±63 ag/ng rRNA), but only once cell line expressed DcR1. Six1 expression (overexpression vs underexpression, mean=108 fg/ng rRNA, range: 0-763) correlated with TRAIL resistance (TRAIL IC_{50}>100 ng/mL, $P=0.05$, χ^2 test) and all cell lines sensitive to TRAIL were also sensitive to anti-DR5

antibody (IC_{50}<1000 ng/mL), but not sensitive to anti-DR4 antibody. Cells were resistant to FasL. Although Six1 mRNA compared across all cell lines did not correlate with DcR2 expression, stable Six1 overexpression in the low-Six1, low-DcR2-expressing CaOV3 cell line increased TRAIL IC_{50} fivefold and significantly increased DcR2, whereas DcR1 levels were unchanged.

Conclusions: Ovarian cancer cells express TRAIL DR4 and DR5 and the decoy receptor DcR2. Decoy receptor DcR1 expression is uncommon. Six1 expression correlates with DcR2 expression and TRAIL resistance in a CaOV3 Six1 over-expression model. The Six1-correlated increase in the TRAIL decoy receptor DcR2 may be a mechanism for TRAIL resistance in ovarian cancers. Given the relationship between Six1 expression and TRAIL resistance, the lack of a direct correlation between Six1 and DcR2 across all cell lines implies other Six1-driven TRAIL resistance mechanisms as well. Additional Six1 overexpression and knockdown experiments are underway.

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The utility of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer

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Objectives: The purpose of this study was to assess the value of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer in complete remission.

Methods: This was a retrospective study of patients with stage IIIC and IV epithelial ovarian cancer diagnosed between 1997 and 2005 who underwent primary surgical debulking followed by adjuvant chemotherapy. We included only patients who had a complete response to adjuvant chemotherapy with no evidence of disease on physical examination, CA-125 determination, and CT scan (when available) and who suffered from recurrence of their disease while under our care.

Results: Seventy-nine patients fit the inclusion criteria. Median age was 59.8 (range: 30-89). Seventy-one patients (89.9%) had stage IIIC and eight (10.1%) had stage IV. Seventy-four patients (93.7%) had papillary serous, two (2.6%) had clear cell, two (2.6%) had endometrioid, and one (1.3%) had mucinous adenocarcinoma. Seventy-seven patients (97.5%) had grade 3, one patient had grade 2, and one had grade 1. Preoperative CA-125 levels were available for 74 patients with a median of 537 (range: 17-25,224) U/mL; six of the 74 had normal preoperative levels (<35 U/mL). The first evidence of recurrence was CA-125 elevation in 62 patients (78.5%), positive clinical findings on physical examination in 9 patients (11.4%), positive CT scan in seven patients (8.9%), and one patient was incidentally found to have recurrent carcinoma in the hernia sac during hernia repair. Of the 9 patients who were first diagnosed with recurrence based on positive clinical findings, seven (77.7%) had significant symptoms that prompted the physical examination (two had bowel obstruction, two had neurologic symptoms, one had flank pain, one had a groin mass, one had a new large breast mass). Two patients had asymptomatic recurrences first found on physical examination during a routine follow-up visit; however, one had an elevated CA-125 and the other had an abnormal CT scan and both of these tests were already scheduled on the same day as the physical exam.

Conclusions: Physical examination has limited utility in detecting ovarian cancer recurrence during routine follow-up visits. Patients with an initial clinically diagnosed recurrence either were symptomatic or had concurrent positive routine CT scan or CA-125. Changing the routinely scheduled follow-up visits to an as-needed basis may be more convenient and economical in patients with epithelial ovarian cancer in remission.

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Therapeutic efficacy of folate receptor α blockade with MORAb-003 in ovarian cancer

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Objectives: The relative overexpression of folate receptor α (FRα) in ovarian cancer compared with normal tissues offers opportunities for novel therapeutic approaches to ovarian cancer. The purpose of this study was to examine the functional significance of FRα blockade with a novel monoclonal antibody, MORAb-003.

Methods: FRα expression was examined in ovarian cell lines (SKOV3ip1, IGROV, HeyA8, A2780-par, and HIO-180) with fluorescence-activated cell sorting analysis. In vitro (cell viability, migration, invasion) and in vivo (tumor growth) effects of FRα blockade on ovarian cancer cells were examined using well-characterized models. The mechanistic effects on the src-family nonreceptor tyrosine kinase Lyn were also examined.

Results: IGROV and SKOV3ip1 cell lines both expressed high levels of FRα compared with the non-transformed (HIO-180) cells. HeyA8 and A2780-par cell lines lacked FRα expression. In vivo, MORAb-003 led to 44 and 84% decreases in tumor growth in SKOV3ip1 and IGROV, respectively, when compared with control IgG antibody. Compared with other groups, the greatest efficacy was noted in the MORAb-003 plus docetaxel group (96 and 99% decreased tumor growth for SKOV3ip1 and IGROV compared with controls, $P<0.001$). In the IGROV model, treatment with MORAb-003 resulted in a 27% decrease in tumor cell proliferation by PCNA staining ($P<0.001$). MORAb-003 redistributed active, phosphorylated Lyn kinase out of lipid rafts, with a 60% decrease in active Lyn compared with control antibody. MORAb-003 did not significantly affect SKOV3ip1 cell viability, migration or invasion in vitro.